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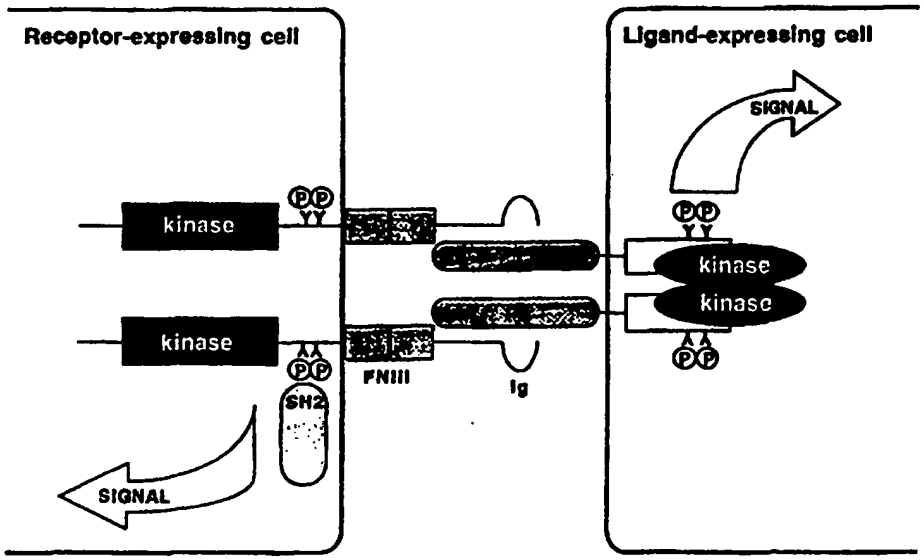

(57) Abstract

A method of modulating the biological activity of a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand, comprising forming a complex between a purified and isolated oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the kinase, and a transmembrane ligand expressed on the cell, thereby affecting a pathway in the cell which is regulated by the transmembrane ligand.

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TITLE: OLIGOMERIZED RECEPTORS WHICH AFFECT PATHWAYS REGULATED BY TRANSMEMBRANE LIGANDS FOR ELK-RELATED RECEPTOR TYROSINE KINASES

FIELD OF THE INVENTION

The invention relates to a method of affecting or modulating a pathway in a cell which is regulated by the binding of a transmembrane ligand for an Elk-related receptor tyrosine kinase and an oligomerized Elk-related receptor tyrosine kinase; a method of identifying substances which affect the binding of a transmembrane ligand for an Elk-related receptor tyrosine kinase and an oligomerized Elk-related receptor tyrosine kinase; and to methods and pharmaceutical compositions using oligomerized Elk-related receptor tyrosine kinases, and substances identified using a method of the invention.

BACKGROUND OF THE INVENTION

Receptor tyrosine kinases play essential roles in cellular signalling events. The largest known family of receptor tyrosine kinases is the Eph subfamily of receptor tyrosine kinases. Eph subfamily tyrosine kinases have been implicated in the control of axon guidance and fasciculation[1-7], in regulating cell migration[8], and in defining compartments in the developing embryo[9-11]. Efficient activation of Eph receptors generally requires that their ligands be anchored to the cell surface, either through a transmembrane (TM) region or a glycosyl phosphatidylinositol (GPI) group[12]. These observations have suggested that Eph receptors can transduce signals initiated by direct cell-cell interactions. Genetic analysis of Nuk, a mouse Eph receptor that binds TM-ligands, has suggested that these ligands have a signalling function[6].

SUMMARY OF THE INVENTION

Challenging cells expressing the transmembrane (TM)-ligands, Elk-L or Htk-L, with the clustered extracellular domain of Nuk was found to induce phosphorylation of the ligands on tyrosine, a process which is mimicked both *in vitro* and *in vivo* by an activated Src tyrosine kinase. Co-culturing of cells expressing a TM-ligand with cells expressing Nuk also was shown to lead to tyrosine phosphorylation of both the ligand and Nuk. Therefore TM-ligands are associated with a tyrosine kinase, and are inducibly phosphorylated upon binding the Nuk receptor, in a fashion reminiscent of cytokine receptors. Furthermore, it was shown that TM-ligands, as well as Nuk, are phosphorylated on tyrosine in mouse embryos, indicating that this is a physiological process. These experimental results confirm that Eph receptors and their TM-ligands mediate bi-directional cell signalling.

Therefore, the present invention provides a method of modulating the biological activity of a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand comprising forming a complex between a purified and isolated oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the Elk-related receptor tyrosine kinase, and the transmembrane ligand expressed on the cell, thereby modulating the biological activity of the transmembrane ligand.

The present invention also provides a method of affecting or modulating a pathway regulated by a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand, comprising forming a complex between a purified and isolated oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the Elk-related receptor tyrosine kinase, and a

transmembrane ligand expressed on the cell, thereby affecting or modulating a pathway in the cell which is regulated by the transmembrane ligand.

The invention also provides a method for evaluating a substance for its ability to modulate the biological activity of a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand comprising the steps of:

(a) contacting an oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the Elk-related receptor tyrosine kinase; a transmembrane ligand which binds to the Elk-related receptor tyrosine kinase to form a receptor-ligand complex, wherein the transmembrane ligand is a transmembrane ligand for an Elk-related receptor tyrosine kinase expressed on a cell; and, a test substance, under conditions which permit the formation of receptor-ligand complexes;

(b) assaying for receptor-ligand complexes, free Elk-related receptor tyrosine kinase, or non-complexed transmembrane ligand, or for activation of the transmembrane ligand; and

(c) comparing to a control to determine if the substance inhibits or enhances the binding of the Elk-related receptor tyrosine kinase and transmembrane ligand, and thereby modulates the biological activity of the transmembrane ligand.

The invention also provides a method for identifying a substance which affects or modulates a pathway regulated by a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand, comprising the steps of:

(a) contacting an oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the Elk-related receptor tyrosine kinase; a transmembrane ligand which binds to the Elk-related receptor tyrosine kinase to form receptor-ligand complexes which activate a pathway regulated by a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand; and a test substance under conditions which permit the formation of receptor-ligand complexes;

(b) assaying for receptor-ligand complexes, free Elk-related receptor tyrosine kinase, or non-complexed transmembrane ligand, or for activation of the transmembrane ligand; and

(c) comparing to a control to determine if the substance inhibits or enhances the binding of the Elk-related receptor tyrosine kinase and transmembrane ligand, and thereby affects or modulates the pathway.

The ability of a substance to inhibit or enhance the binding of an oligomerized Elk-related receptor tyrosine kinase and transmembrane ligand correlates with the ability of the substance to inhibit or enhance the biological activity of the transmembrane ligand, including the signal transduction activities of the ligand, and in particular the activation of a pathway regulated by the ligand.

The invention also contemplates a method for evaluating a substance for its ability to inhibit or enhance the interaction of an oligomerized Elk-related receptor tyrosine kinase, or an isoform, or an extracellular domain of the Elk-related receptor tyrosine kinase, and a transmembrane ligand for an Elk-related receptor tyrosine kinase expressed on a cell comprising the steps of:

(a) providing a reporter gene operably linked to a DNA binding site for a transcriptional activator;

(b) providing a first hybrid protein comprising the transmembrane ligand in polypeptide linkage to a DNA binding domain of the transcriptional activator;

(c) providing a second hybrid protein comprising an oligomerized Elk-related receptor tyrosine kinase or an isoform or an extracellular domain of the Elk-related receptor tyrosine kinase in polypeptide linkage to an activation domain of the transcriptional activator; under conditions where the transmembrane ligand and oligomerized Elk-related receptor tyrosine kinase can bind and thereby reconstitute the transcriptional activator which induces transcription of the reporter gene;

(d) administering a test substance; and

(e) monitoring expression of the reporter gene, wherein a decrease in expression is an indication that the substance inhibits the interaction of the transmembrane ligand and oligomerized Elk-related receptor tyrosine kinase, and an increase in expression is an indication that the substance enhances the interaction of the transmembrane ligand and oligomerized Elk-related receptor tyrosine kinase. In an alternate method, the oligomerized Elk-related receptor tyrosine kinase is linked to the DNA binding domain, and the transmembrane ligand is linked to the activation domain.

In another aspect, the invention features an antibody preparation which specifically binds to a receptor-ligand complex comprising an oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the kinase, and a transmembrane ligand for an Elk-related receptor tyrosine kinase.

In another aspect, the invention features a method of purifying a compound which inhibits or enhances the binding of an oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the kinase, and a transmembrane ligand for an Elk-related receptor tyrosine kinase comprising contacting the compound with one of the ligand or receptor; and, isolating the compound by its binding affinity for the ligand or receptor.

The substances, and compounds obtained using the methods of the invention and the antibodies specific for receptor-ligand complexes may be used to modulate the biological activity of a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand, including inhibiting or enhancing signal transduction activities of the transmembrane ligand, and in particular modulating a pathway regulated by the transmembrane ligand.

The invention still further provides a method for affecting or modulating neuronal development or regeneration in a subject comprising administering to a subject an effective amount of a purified and isolated oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain thereof, a substance or compound identified using a method of the invention, or an antibody specific for a receptor-ligand complex of the invention.

In yet another aspect, the invention provides a method for affecting or modulating axonogenesis in a subject comprising administering to a subject an effective amount of a purified and isolated oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain thereof, a substance or compound identified using a method of the invention, or an antibody specific for a receptor-ligand complex of the invention.

The invention also relates to a pharmaceutical composition which comprises a purified and isolated oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain thereof, a substance or compound identified using a method of the invention, or an antibody specific for a receptor-ligand complex

of the invention, in an amount effective to stimulate or inhibit neuronal development or regeneration and a pharmaceutically acceptable carrier, diluent or excipient.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to the drawings in which:

Figure 1A is an alignment of human Elk-L, Htk-L and Elk-L3 cytoplasmic domains;

Figure 1B is an immunoblot showing phosphorylation of GST-hElk-L cytoplasmic domain fusion protein (GST-Elk-L cyt) *in vitro* by v-Src;

Figure 1C is an immunoblot showing expression of Elk-L and Htk-L either alone or with v-Src in Cos-1 cells;

Figure 1D is an immunoblot showing expression of TM-ligands either alone or with v-Src in Cos-1 cells;

Figure 2A are immunoblots showing induction of tyrosine phosphorylation of TM-ligands, expressed in Cos-1 cells, upon stimulation with clustered Nuk-Fc;

Figure 2B is an immunoblot showing stimulation of Elk-L tyrosine phosphorylation by Nuk-Fc in CHP-100 cells, which express endogenous Elk-L;

Figure 2C are immunoblots showing bi-directional signalling between Nuk-expressing NG108 cells and TM-ligand-expressing cells in co-culture;

Figure 3 is an anti-phosphotyrosine immunoblot of TM-ligands and Nuk receptor immunoprecipitated from E10.5 mouse body tissue;

Figure 4 is a schematic diagram showing bi-directional signalling by Nuk and its TM-ligands;

Figure 5A is a composite sequence of cDNAs encoding full length human Elk-L3 and a segment of rat Elk-L3;

Figure 5B shows amino acid sequences of all the known Eph family ligands aligned with each other, using the human versions with the exception of mouse ELF-1; and

Figure 5C shows in matrix form the percent similarity between ligands in Figure 5B.

DETAILED DESCRIPTION OF THE INVENTION

As hereinbefore mentioned, the present invention provides a method of modulating the biological activity of a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand comprising forming a complex between a purified and isolated oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the kinase, and a transmembrane ligand expressed on the cell. The biological activity of a transmembrane ligand may be modulated by inhibiting or enhancing the signal transduction activities of the ligand including affecting or modulating a pathway regulated by a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane

ligand.

A pathway which is regulated by a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand refers to a regulatory pathway in the cell that affects, for example gene expression, cell division, cytoskeletal architecture, cell metabolism, migration, cell-cell interactions, and spatial positioning, and which is activated by the binding of transmembane ligands with an oligomerized Elk-related receptor tyrosine kinase, or an isoform, or an extracellular domain thereof. Examples of such pathways are the GAP/Ras pathway, the pathway that regulates the breakdown of the polyphosphoinositides through phospholipase C (PLC) and the Src/tyrosine kinase and Ras pathways.

"Transmembrane ligand or transmembrane ligands" refers to a class of ligands which are anchored to the cell membrane through a transmembrane domain, and bind to the extracellular domain of an Eph subfamily receptor tyrosine kinase, facilitating dimerization and autophosphorylation of the receptor. The transmembrane ligands which are targeted or used in accordance with the methods of the invention are those that bind to and preferably activate (i.e. phosphorylate) an Elk-related receptor tyrosine kinase (see discussion of the Elk-related receptor tyrosine kinases below). Preferably the transmembrane ligands used in the methods of the invention are Elk-L/LERK2/Efl-3/Cek5-L; hHtk-L/ELF-2/Lerk5 (Tessier-Lavigne, M., 1995, Cell 82:345-348) and hElk-L3/Efl-6 (Gale et al.). These transmembrane ligands have a highly conserved cytoplasmic region with multiple potential sites for tyrosine phosphorylation[12-17]. The amino acid sequences for the transmembrane ligands hElk-L3, hHtk-L, and hElk-L, and the cytoplasmic, transmembrane, and extracellular domains of the ligands are shown in Figure 5, which is Figure 1 in Gale, N.W. et al., 1996.

Transmembrane ligands which may be selected or targeted in accordance with the present invention also include (i) proteins having sequence identity with known transmembrane ligands such as hElk-L3, hHtk-L, and hElk-L, and their homologs; (ii) proteins which can bind with the extracellular domain of an Elk-related receptor tyrosine kinase; and (iii) chimeric proteins of transmembrane ligands, e.g. a protein containing a cytoplasmic domain of one transmembrane ligand and an extracellular domain from a different transmembrane ligand.

The term "Elk-related receptor tyrosine kinase" refers to a particular subclass of the Eph subfamily of receptor tyrosine kinases. The Eph subfamily receptor tyrosine kinases are a closely related group of transmembrane receptor tyrosine kinases which contain cell adhesion-like domains on their extracellular surface. The Eph subfamily receptor tyrosine kinases are more specifically characterised as encoding a structurally related cysteine rich extracellular domain containing a single immunoglobulin (Ig)-like loop near the N-terminus and two fibronectin III (FN III) repeats adjacent to the plasma membrane. For example, Nuk contains 20 cysteine residues whose position is conserved in the extracellular domain of Eph family members, an immunoglobulin-like domain near the amino terminus (Ig-like), and two fibronectin type III repeats (FN III; between Nuk amino acids residues 330-420 and 444-534). The Ig-like domain of Nuk contains specific residues (Cys^{70}, Trp^{80}, Cys^{115}) known to be conserved in the Ig superfamily (Williams and Barclay, *Ann. Rev. Immunol.* 6:381-405, 1988).

The Elk-related receptor tyrosine kinases bind to, and are phosphorylated by transmembrane ligands, and include mouse Nuk and its homologs namely, Hek5 and Erk in humans, Sek3 in mice, and Cek5 in

chickens; rat Elk and its homologs including Cek6a in chickens and xEK; human Hek2 and its homologs including Sek4 in mice and Cek10 in chickens; and human Htk and its homologs including Mykl in mice. The amino acid sequences for representative Elk-related receptor tyrosine kinases can be found in GenBank, for example Accession Nos. L25890 (Nuk), X13411 (rat Elk), U07695 (human Htk), M83941 (Hek), and the publications referred to therein.

Homologs of Elk-related receptor tyrosine kinases are identified by aligning a sequence of a putative homolog with a known Elk-related receptor tyrosine kinase and comparing the positions in each sequence. When a position in the compared sequence is occupied by the same nucleotide base or amino acid, then the molecules are matching or have identical positions shared by the sequences. Kinase proteins which may be used in the methods and compositions of the invention may have over 60%, preferably over 70%, most preferably over 80% identity with an Elk-related receptor tyrosine kinase.

Isoforms of an Elk-related receptor tyrosine kinase may be used in the methods and compositions of the invention. Generally, an isoform contains the same number and kinds of amino acids and it binds to a transmembrane ligand as described herein, but the isoform has a different molecular structure.

An extracellular domain of an Elk-related receptor tyrosine kinase may also be used in the methods and compositions of the invention. The extracellular domain of an Elk-related receptor tyrosine kinase is generally defined as the region extracellular to the transmembrane domain. Specifically it is characterised by a cysteine rich region, whose position is conserved in the extracellular domain of Eph receptor family members, an immunoglobulin-like domain near the amino terminus (Ig-like), and two fibronectin type III repeats (FN III). An extracellular domain of an Elk-related receptor tyrosine kinases may be selected based on these characteristic features and by comparing the amino acid sequences of the extracellular domains of known Elk-related receptor tyrosine kinases.

An Elk-related receptor tyrosine kinase may be selected for use in a method or composition of the invention based on the nature of the transmembrane ligand which is targeted or selected. The selection of a specific complementary ligand and Elk-related receptor tyrosine kinase (e.g. Elk-L and Elk or Nuk; and Htk-L and Elk or Nuk) in a method of the invention may allow for the identification of a specific substance that affects a pathway regulated by a specific transmembrane ligand.

An Elk-related receptor tyrosine kinase or extracellular domain thereof, or a transmembrane ligand may be isolated from cells which are known to express the proteins. Alternatively the protein or part of the protein may be prepared using conventional recombinant DNA methods (e.g. baculovirus expression in insect cells). The proteins or parts thereof may also be prepared by chemical synthesis using standard techniques such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

The Elk-related receptor tyrosine kinase protein or extracellular domain thereof, or transmembrane ligands may also be expressed on the surface of a cell (e.g. Cos-1 cell) using conventional methods.

An Elk-related receptor tyrosine kinase or extracellular domain thereof, or transmembrane ligand may be conjugated with other molecules, such as proteins or polypeptides. For example, N-terminal fusion proteins

may be synthesized by fusing, through recombinant techniques, the N-terminal of an Elk-related receptor tyrosine kinase or extracellular domain thereof, and the sequence of a selected protein or marker protein with a desired biological function, such as an oligomerization motif which facilitates oligomerization of the receptor or ligand. Examples of oligomerization motifs include immunoglobulins, and parts thereof such as the constant region of an immunoglobulin; and motifs which introduce reactive groups which provide for hydrophobic interactions between receptors or ligands, for example, amphoteric α-helices.

The transmembrane ligands and Elk-related receptor tyrosine kinase or extracellular domain thereof, or fusions thereof, used in the methods and compositions of the invention are oligomerized. An Elk-related receptor tyrosine kinase or extracellular domain thereof, or transmembrane ligands may be dimerized by preparing fusion proteins as discussed above containing an oligomerization motif such as a constant region of an immunoglobulin. Clusters of receptors or ligands may then optionally be prepared by adding antibodies specific for the constant region of the immunoglobulin. For example, a Nuk extracellular domain-IgG constant chain fusion protein may be clustered using anti-human IgG. If the receptor tyrosine kinase or transmembrane ligand is associated with a cell, interaction of the receptor or extracellular domain thereof with a transmembrane ligand will result in dimerization of the receptor or ligand.

The invention provides a method for evaluating a substance for its ability to modulate the biological activity of a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand. The method involves contacting an oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the Elk-related receptor tyrosine kinase; a transmembrane ligand for an Elk-related receptor tyrosine kinase expressed on a cell that binds to the Elk-related receptor tyrosine kinase to form a receptor-ligand complex; and, a test substance, under conditions which permit the formation of receptor-ligand complexes. Receptor-ligand complexes, free Elk-related receptor tyrosine kinase, or non-complexed transmembrane ligand, or activation of the transmembrane ligand are assayed. The results are compared to a control to determine if the substance inhibits or enhances the binding of the Elk-related receptor tyrosine kinase and transmembrane ligand, and thereby modulates the biological activity of the transmembrane ligand.

In an embodiment, the invention provides a method for identifying a substance which affects or modulates a pathway regulated by a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand. An oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the Elk-related receptor tyrosine kinase, is contacted with a test substance, and a transmembrane ligand which binds to the receptor to form receptor-ligand complexes which activate the pathway, under conditions which permit the formation of receptor-ligand complexes. Receptor-ligand complexes, free Elk-related receptor tyrosine kinase, or non-complexed transmembrane ligands, or activation of the ligand are assayed and the results are compared to a control to determine the effect of the substance.

A substance identified using a method of the invention may stimulate or inhibit the binding of an oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain thereof, and transmembrane ligands, or compete for a site on the oligomerized Elk-related receptor tyrosine kinase which binds the transmembrane ligands or a site on the transmembrane ligands which binds to the oligomerized Elk-

related receptor tyrosine kinase. The substance may be an endogenous physiological substance, or it may be a natural or synthetic drug.

Oligomerized Elk-related receptor tyrosine kinases that may be used in the methods of the invention are described herein. In particular, an oligomerized extracellular domain of an Elk-related receptor tyrosine kinase, preferably Nuk, can be employed in the method. The oligomerized extracellular domain may also be prepared as a fusion protein as described herein. The Elk-related receptor tyrosine kinase may be associated with a cell which either exogenously expresses the kinase (e.g. transformed Cos-1 cells which express the kinase) or a cell which endogenously expresses the kinase (e.g. Cos-1 monkey kidney cells endogenously expressing Nuk).

The transmembrane ligand used in the methods of the invention may be a ligand which is native to the selected oligomerized Elk-related receptor tyrosine kinase, or it may be a ligand which is not native to the selected receptor tyrosine kinase. The transmembrane ligand is preferably associated with a cell which either exogenously expresses the ligand (e.g. transformed Cos-1 cells) or a cell which endogenously expresses the ligand (e.g. CHP-100 cells which express the transmembrane ligand Elk-L).Where the transmembrane ligand used in a method of the invention is not cell associated, it should be oligomerized using the methods described herein.

Conditions which permit the formation of receptor-ligand complexes may be selected having regard to factors such as the nature and amounts of the receptor and the ligand.

The receptor-ligand complex, free oligomerized receptor or non-complexed transmembrane ligand may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof.

Antibody against the ligand or the receptor, or a labelled ligand, or a labelled oligomerized receptor may be utilized in the methods of the invention to facilitate isolation of the complexes etc. The antibodies, the oligomerized receptor, or substance may be labelled with a detectable substance.

The receptor or ligand used in the method of the invention may be insolubilized. For example, the receptor or ligand may be bound to a suitable carrier including agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc. The insolubilized receptor or ligand may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

Where the ligand is expressed on the surface of a cell the affect of a test substance may be determined by assaying for activation of the ligand, or by assaying for a biological affect on the cell. Activation of the ligand may be determined by assaying for phosphorylation of the ligand.

The interaction of the receptor and ligand may be identified using a two-hybrid expression system wherein the activity of a transcriptional activator is reconstituted.(See for example, Chien et al. 1991, Proc. Natl. Acad. Sci. (USA) 88:9578 re two-hybrid systems). The system may comprise a reporter gene (e.g. genes

encoding gene products such as β-galactosidase (e.g. lacZ), luciferase, alkaline phosphatase, horseradish peroxidase), operably linked to a DNA binding site for a transcriptional activator; a first hybrid protein comprising a transmembrane ligand for an Elk-related receptor tyrosine kinase in polypeptide linkage to a DNA binding domain of a transcriptional activator; and a second hybrid protein comprising an oligomerized Elk-related receptor tyrosine kinase or an isoform or an extracellular domain of the Elk-related receptor tyrosine kinase, in polypeptide linkage to an activation domain of the transcriptional activator. The system is carried out employing conditions that allow binding of the transmembrane ligand and oligomerized Elk-related receptor tyrosine kinase thereby reconstituting the transcriptional activator which induces transcription of the reporter gene. A test substance is added, and the expression of the reporter gene is monitored. A decrease in expression is an indication that the substance inhibits the interaction of the transmembrane ligand and oligomerized Elk-related receptor tyrosine kinase, and an increase in expression is an indication that the substance enhances the interaction of the transmembrane ligand and oligomerized Elk-related receptor tyrosine kinase. In an alternate method, the oligomerized Elk-related receptor tyrosine kinase is linked to the DNA binding domain, and the transmembrane ligand is linked to the activation domain.

The invention also provides host organisms (typically unicellular organisms) which harbor a two-hybrid system as described herein. Usually the host organism is a yeast cell such as *Saccharomyces cervisiae*.

In a particular system, the yeast GAL4 protein which has a domain responsible for DNA-binding and another domain for transcriptional activation is employed. In the expression system, plasmids encoding two hybrid proteins one containing the GAL4 DNA binding domain fused to a first protein (a transmembrane ligand or an oligomerized Elk-related receptor tyrosine kinase), and a second plasmid containing the GAL4 activation domain fused to a second protein (a transmembrane ligand or an oligomerized Elk-related receptor tyrosine kinase which forms a complex with the first protein) are introduced into the yeast. If the first and second proteins interact with one another, the ability to activate transcription from promoters containing GAL4-binding sites is reconstituted leading to the expression of a reporter gene e.g. lacZ.

The invention also features an antibody preparation which specifically binds to a receptor-ligand complex comprising an oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the kinase, and a transmembrane ligand for an Elk-related receptor tyrosine kinase. Antibodies (e.g. monoclonal and polyclonal antibodies) may be prepared against oligomerized receptor-ligand complexes. The invention can employ not only intact monoclonal or polyclonal antibodies, but also immunologically active fragments (e.g. a Fab or $(Fab)_2$ fragment), an antibody heavy chain, and antibody light chain, a genetically engineered single chain F_v molecule (Ladner et al, U.S. Pat. No. 4,946,778), or a chimeric antibody, for example, an antibody which contains the binding specificity of a murine antibody, but in which the remaining portions are of human origin. Antibodies including monoclonal and polyclonal antibodies, fragments and chimeras, may be prepared using methods known to those skilled in the art.

The invention also features a method of purifying a compound which inhibits or enhances the binding of an oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the kinase, and a transmembrane ligand for an Elk-related receptor tyrosine kinase comprising contacting the compound with one of the ligand or receptor; and, isolating the compound by its binding affinity for the ligand or receptor.

Conventional affinity binding methods can be used to isolate a compound.

The substances and compounds obtained using the methods of the invention, and the antibodies specific for receptor-ligand complexes may be used to modulate the biological activity of a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand, including inhibiting or enhancing signal transduction activities of the transmembrane ligand, and in particular modulating a pathway in a cell regulated by a transmembrane ligand for an Elk-related receptor tyrosine kinase, particularly those pathways involved in neuronal development, axonal migration, pathfinding and regeneration. The identification and isolation of substances and compounds will permit studies of the role of the substances and compounds in the developmental regulation of axonogenesis and neural regeneration, and permit the development of substances which affect these roles, such as functional or non-functional analogues of the oligomerized extracellular domain of an Elk-related receptor tyrosine kinase. The substances, compounds,and substances derived therefrom, and antibodies, will be useful as pharmaceuticals to modulate axonogenesis, nerve cell interactions and regeneration, to treat conditions such as neurodegenerative diseases and conditions involving trauma and injury to the nervous system, for example Alzheimer's disease, Parkinson's disease, Huntington's disease, demylinating diseases, such as multiple sclerosis, amyotrophic lateral sclerosis, bacterial and viral infections of the nervous system, deficiency diseases, such as Wernicke's disease and nutritional polyneuropathy, progressive supranuclear palsy, Shy Drager's syndrome, multistem degeneration and olivo ponto cerebellar atrophy, peripheral nerve damage, trauma and ischemia resulting from stroke.

The present invention thus provides a method for affecting neuronal development or regeneration in a subject comprising administering to a subject an effective amount of a purified and isolated oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain thereof, a substance or compound identified using a method of the invention, or antibodies specific for oligomerized receptor-ligand complexes. The invention also contemplates a method for stimulating or inhibiting axonogenesis in a subject comprising administering to a subject an effective amount of a purified and isolated oligomerized Elk-related receptor tyrosine kinase protein, or an isoform or an extracellular domain thereof, a substance or compound identified using a method of the invention, or antibodies specific for oligomerized receptor-ligand complexes.

The invention still further relates to a pharmaceutical composition which comprises a purified and isolated oligomerized Elk-related receptor tyrosine kinase protein or an isoform or an extracellular domain thereof, a substance or compound identified using a method of the invention, or antibodies specific for oligomerized receptor-ligand complexes, in an amount effective to regulate neuronal development or regeneration and a pharmaceutically acceptable carrier, diluent or excipient. The pharmaceutical compositions may be used to stimulate or inhibit neuronal development, regeneration and axonal migration associated with neurodegenerative conditions and conditions involving trauma and injury to the nervous system as described above.

The compositions of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the protein to be administered in which any toxic effects are outweighed by the therapeutic effects of the protein. The term subject is intended to include mammals and

includes humans, dogs, cats, mice, rats, and transgenic species thereof. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of an oligomerized Elk-related receptor tyrosine kinase protein may vary according to factors such as the condition, age, sex, and weight of the individual. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active compound (e.g., protein) may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or intracerebral administration. Preferably, the pharmaceutical compositions of the invention are administered directly to the peripheral or central nervous system, for example by administration intracerebrally.

A pharmaceutical composition of the invention can be administered to a subject in an appropriate carrier or diluent, co-administered with enzyme inhibitors or in an appropriate carrier such as microporous or solid beads or liposomes. The term "pharmaceutically acceptable carrier" as used herein is intended to include diluents such as saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Strejan et al., (1984) J. Neuroimmunol 7:27). The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms. Depending on the route of administration, the active compound may be coated to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

The pharmaceutical compositions may be administered locally to stimulate axonogenesis and pathfinding, for example the compositions may be administered in areas of local nerve injury or in areas where normal nerve pathway development has not occurred. The pharmaceutical compositions may also be placed in a specific orientation or alignment along a presumptive pathway to stimulate axon pathfinding along that line, for example the pharmaceutical compositions may be incorporated on microcarriers laid down along the pathway. In particular, the pharmaceutical compositions of the invention may be used to stimulate formation of connections between areas of the brain, such as between the two hemispheres or between the thalamus and ventral midbrain. The pharmaceutical compositions may be used to stimulate formation of the medial tract of the anterior commissure or the habenular interpeduncle.

Therapeutic administration of polypeptides may also be accomplished using gene therapy, A nucleic acid including a promoter operatively linked to a heterologous polypeptide may be used to produce high-level expression of the polypeptide in cells transfected with the nucleic acid. DNA or isolated nucleic acids may be introduced into cells of a subject by conventional nucleic acid delivery systems. Suitable delivery systems include liposomes, naked DNA, and receptor-mediated delivery systems, and viral vectors such as retroviruses, herpes viruses, and adenoviruses.

The following non-limiting example is illustrative of the present invention:

EXAMPLE

The following is a detailed description of Figures 1 to 4 including a detailed description of the methods utilized in the experiments illustrated in the Figures and outlined in the Example:

Phosphorylation of transmembrane ligands by v-Src. (Figure 1A) Alignment of human Elk-L, Htk-L and Elk-L3 cytoplasmic domains. Black boxes indicate residues conserved in all TM-ligands; grey boxes indicate residues conserved in two ligands. Conserved tyrosines are marked by an asterisk. Mouse Htk-L cytoplasmic domain is identical to human Htk-L except for a serine to glycine substitution at position 278. Elk-L is also referred to as Lerk-2[13] and Cek5-L[14], Htk-L as Lerk-5[25] and Elf-2[15]. **(Figure 1B)** Phosphorylation of GST-hElk-L cytoplasmic domain fusion protein (GST-Elk-L cyt) in vitro by v-Src. v-Src was immunoprecipitated from v-Src transformed Rat-2 cells and incubated with GST fusion proteins or enolase as exogenous substrates in the presence of $^{32}P\gamma ATP$. **(Figure 1C)** and **(Figure 1D)** Tyrosine phosphorylation of TM-ligands upon coexpression with v-Src. **(Figure 1C)** Elk-L and Htk-L were expressed either alone or with v-Src in Cos-1 cells and immunoprecipitated with anti-ligand antibody. Upper panel: anti-phosphotyrosine blot; lower panel: anti-ligand blot (reprobe). **(Figure 1D)** TM-ligands were expressed as in **(Figure 1C)**, precipitated using a Nuk extracellular domain IgG fusion protein (Nuk-Fc) as an affinity reagent, and immunoblotted with anti-phosphotyrosine serum. The band observed at ~100 kDa represents cross-reaction of Nuk-Fc with the protein A-HRP. **Methods: (Figure 1B)** v-Src was immunoprecipitated from v-Src transformed Rat-2 cells using an anti-Src monoclonal antibody (Oncogene Science) and immune complexes were incubated for 15 minutes at RT with 5 μCi of $^{32}P\gamma ATP$ in Src-KRB[26] alone or in buffer containing enolase, 10 μg purified GST or GST-Elk-L cyt (residues 262-343 of hElk-L[12]) as exogenous substrates. Proteins were separated on a 10% SDS-PAGE gel and ^{32}P labelled proteins were detected by autoradiography. **(Figure 1C)** and **(Figure 1D)** Cos-1 cells were transiently transfected as indicated with 5 μg of hElk-L, mHtk-L or v-Src cDNA expression vectors, either alone or in combination, or with empty control vector. Cells were serum starved for approximately 20 hours in medium containing 0.5% foetal bovine serum (FBS) and lysed in PLC lysis buffer[2] at approximately 60 hours post transfection. TM-ligands were precipitated using **(Figure 1C)** anti-ligand serum (raised against residues 326-343 of hElk-L, which also recognises Htk-L; Santa Cruz) or **(Figure 1D)** 10 μg of Nuk-Fc fusion protein[11] plus protein A sepharose. Precipitated proteins were washed three times in HNTG[2], separated on a 10% SDS-PAGE gel, transferred to PVDF membrane (Millipore) and immunoblotted with **(Figure 1C)** monoclonal (4G10) or **(Figure 1D)** polyclonal anti-phosphotyrosine antibodies. Detection was by Enhanced Chemiluminescence (Pierce). In **(Figure 1C)** the filter was stripped using 0.1 M glycine pH 2.5 and reprobed with anti-ligand serum.

Stimulation of tyrosine phosphorylation of TM-ligands by Nuk extracellular domain and Nuk-expressing cells.

Figure 2A: *Induction of tyrosine phosphorylation of TM-ligands, expressed in Cos-1 cells, upon stimulation with clustered Nuk-Fc.* Cos-1 cells were transiently transfected with Elk-L, Htk-L, or control expression vectors and treated with 2 μg/ml clustered Nuk-Fc fusion protein or Fc tag alone (c) for the indicated periods of time. Cells were lysed, immunoprecipitated with anti-ligand serum and blotted with antibodies to phosphotyrosine (upper panels). Filters were stripped and reprobed with anti-ligand serum (lower panels). As a control, excess

immunizing peptide was included as indicated (+ pep). Elk-L and Htk-L phosphorylated by cotransfection with v-Src were included on these gels to indicate the mobility of tyrosine phosphorylated ligands; as less protein was loaded in these lanes than the Nuk-Fc stimulated lanes, no bands were detected upon reprobing with anti-ligand serum.

Figure 2B: *Stimulation of Elk-L tyrosine phosphorylation by Nuk-Fc in CHP-100 cells, which express endogenous Elk-L.* Cells were stimulated with 2 µg/ml clustered Nuk-Fc or Fc alone (c) for the indicated periods of time, lysed and immunoblotted as in **Figure 2A**. Upper panel: anti-phosphotyrosine blot; lower panel: anti-ligand blot (reprobe).+ Pep = + competing immunizing peptide.

Figure 2C: *Bi-directional signalling between Nuk-expressing and TM-ligand-expressing cells in co-culture.* Cos-1 cells transiently transfected with Elk-L or Htk-L were co-cultured with parental or Nuk-expressing NG108 cells for the indicated times. Left panels: anti-ligand IP; right panels: anti-Nuk IP from pooled, cocultured cells. Upper panels: anti-phosphotyrosine blots; lower panels: anti-ligand blots (reprobe). **Methods:** In **Figure 2A** and **Figure 2B**, Cos-1 cells were transiently transfected and serum starved as in Figure 1. Human neuroepithelial CHP-100 cells were serum starved for 8 hours in medium containing 0.5% FBS. Nuk-Fc[11] or Fc tag (c) was clustered using anti-human IgG (Jackson Immunoresearch) for 1-2 hours at 4°C, diluted to a final concentration of 2 µg/ml in serum free medium, and applied to cells for the indicated periods of time. Cells were lysed in PLC lysis buffer and immunoprecipitated with anti-ligand antibodies. Immunoprecipitation of TM-ligands was inhibited where indicated by addition of 100-fold excess immunizing Elk-L C-terminal peptide (+ pep; residues 326-343 of hElk-L, Santa Cruz). Immune complexes were separated and transferred as in Figure 1 and immunoblotted with monoclonal anti-phosphotyrosine antibodies (4G10; upper panel). In **Figure 2C**, NG108-15 cells (NG108: mouse neuroblastoma x rat glioma fusion[20]) were stably transfected with an expression vector containing full-length Nuk, and indvidual G418 resistant clones were isolated (NG108-Nuk). Parental or Nuk-expressing NG108 cells were removed from the plate by tituration and resuspended in PBS + magnesium and calcium. Cell suspensions were placed on top of serum starved Cos-1 cells transiently expressing Elk-L (left panels) or Htk-L (right panels). Cells were cocultured for 30 or 60 minutes at 37°C, 5% CO_2 and lysed together in PLC lysis buffer. Cleared lysates were divided in two and immunoprecipitated with either anti-ligand or anti-Nuk serum[2] as indicated. Proteins were separated and transferred as in Figure 2 and immunoblotted with monoclonal anti-phosphotyrosine antibodies (upper panels). Blots were stripped and reprobed with anti-ligand antibodies (lower panel).

Figure 3. Both transmembrane ligands and Nuk are phosphorylated on tyrosine in the mouse embryo. Anti-phosphotyrosine immunoblot of TM-ligands (left panel) and Nuk receptor (right panel) immunoprecipitated from E10.5 mouse body tissue. Lysed tissue was immunoprecipitated with anti-Nuk or pre-immune serum (PI), or anti-ligand antibodies with or without addition of excess competing immunizing peptide (ligand pep). The mobility of tyrosine phosphorylated Elk-L and Htk-L is indicated by inclusion of v-Src phosphorylated TM-ligands on the gel (Elk-L + v-Src and Htk-L + v-Src). **Methods:** E10.5 mouse embryos from wild-type matings were harvested and divided into heads and bodies. Tissue was lysed in PLC lysis buffer by Dounce homogenisation, cleared and precleared by incubation with protein A sepharose. Supernatants were immunoprecipitated with anti-Nuk or pre-immune serum, or anti-ligand antibodies with or

without addition of 100-fold excess competing ligand C-terminal peptide. Proteins were separated, transferred and immunoblotted with monoclonal antiphosphotyrosine antibodies. Results for E10.5 head tissue were essentially identical.

Figure 4. Model for bi-directional signalling by Nuk and its TM-ligands. Interaction of the ligand-expressing cell (right) with the receptor-expressing cell (left) promotes aggregation and autophosphorylation of the receptor. This is followed by recruitment of SH2 domain-containing proteins to phosphorylated tyrosines e.g. in the juxtamembrane region[27, 28], and tyrosine phosphorylation of cellular proteins. Concomitantly, interaction of the receptor with TM-ligands causes ligand clustering and phosphorylation by an associated tyrosine kinase, leading to propagation of signals in the ligand presenting cell.

Description of Results:

Nuk belongs to a subclass of Eph receptors that bind specifically to the TM-subgroup of Eph receptor ligands[11, 14, 19]. Genetic analysis of *Nuk* in the mouse has revealed a physiological role for this receptor in pathfinding of specific anterior commissure axons, and has raised the possibility that the TM-ligands might themselves possess a signalling function, which is activated by binding of the Nuk extracellular domain[6]. The three known TM-ligands have highly conserved cytoplasmic domains, and are virtually identical over their C-terminal 33 amino acids[12-17]. These sequences contain five potential tyrosine phosphorylation sites (Figure 1A). A GST fusion protein containing the cytoplasmic domain of human Elk-L (residues 262-343) was tyrosine phosphorylated by v-Src in vitro (Figure 1B), whereas GST alone or a fusion protein containing the Elk-L extracellular domain were not (Figure 1B and data not shown).

To investigate whether full-length Elk-L or Htk-L could be phosphorylated on tyrosine in vivo, these TM-ligands were expressed in Cos-1 cells either alone or in combination with v-Src. The ligands were then precipitated from the transfected cells using either an antibody to the common C-terminal region of Elk-L and Htk-L (anti-ligand) or a fusion protein containing the extracellular domain of Nuk fused to the Fc region of the Ig heavy chain (Nuk-Fc[11]), which binds with high affinity to the extracellular domain of TM-ligands. When such precipitates were blotted with ligand antibody, a diffuse band of approximately 45-48 kDa was specifically detected in cells transfected with Elk-L, whereas a protein of between 38 and 46 kDa was identified in cells transfected with Htk-L (Figure 1C lower panel). The predicted molecular weights of Elk-L and Htk-L are 38 and 37 kDa respectively, and their slow electrophoretic mobility is apparently due to glycosylation (data not shown). Immunoblotting of anti-ligand immunoprecipitates from transfected Cos-1 cells with antibodies to phosphotyrosine (Figure 1C upper panel) showed that both Elk-L and Htk-L were basally phosphorylated on tyrosine at low levels. Co-transfection of Elk-L with v-Src led to the appearance of a highly tyrosine phosphorylated ~48 kDa form of Elk-L in both anti-ligand and Nuk-Fc precipitates (Figure 1C upper panel and Figure 1D). In addition a tyrosine phosphorylated protein of 130-140 kDa was observed to co-precipitate with Elk-L from cells co-expressing ligand and v-Src. v-Src also induced strong tyrosine phosphorylation of Htk-L, which migrated as a broad band of 38-48 kDa in the phosphorylated form. Immunoprecipitation of both Elk-L/Htk-L and the 130 kDa protein was markedly reduced by addition of the immunizing peptide which competes for antibody binding (data not shown). In v-Src co-transfected cells the total amount of either ligand detected in western blots by the anti-ligand antibodies was reduced. It is possible that these antibodies, which

were raised against the C-terminal part of Elk-L, are less efficient in recognising highly tyrosine phosphorylated forms of the denatured ligands in an immunoblot. These results show that Elk-L and Htk-L are potent *in vivo* substrates for an activated Src tyrosine kinase, and can be detected in association with other phosphotyrosine-containing proteins in cells expressing both ligand and v-Src.

The phosphorylation of TM-ligands on tyrosine may provide a mechanism by which signals are transmitted into ligand-presenting cells and such a signal might be activated by the clustering of ligands on the cell surface. To address this issue, Cos-1 cells expressing Elk-L or Htk-L were exposed to the Nuk extracellular domain, in the form of a Nuk-Fc fusion protein clustered with anti-Ig. Under these conditions Nuk-Fc induced a several-fold increase in the tyrosine phosphorylation of both Elk-L and Htk-L, whereas no stimulation of ligand tyrosine phosphorylation was induced by Fc alone (Figure 2A). The tyrosine phosphorylated band immunoprecipitated by the anti-ligand serum was markedly reduced by addition of excess ligand C-terminal peptide to the immunoprecipitates. This experiment indicates that the binding of clustered Nuk-Fc to the TM-ligands induces the activation of an endogenous tyrosine kinase in Cos-1 cells that can subsequently phosphorylate Elk-L and Htk-L. These results, whilst provocative, were performed using exogenously overexpressed ligand. To corroborate these observations in a more physiologically relevant cell type, the human neuroepithelioma cell line CHP-100, previously shown to express endogenous Elk-L[12] was employed. Incubation of CHP-100 cells with clustered Nuk-Fc led to a striking increase in the tyrosine phosphorylation of Elk-L and to coprecipitation of several tyrosine phosphorylated polypeptides (Figure 2B). Thus, the binding of the Nuk extracellular domain to a cell that normally expresses Elk-L also leads to tyrosine kinase activation and concomitant Elk-L phosphorylation.

These findings raised the possibility that the interaction of a cell expressing TM-ligands on its surface with a second cell expressing Nuk might lead to both the activation of the Nuk receptor, and subsequent signalling within the Nuk-expressing cell, and also to the activation of a ligand-associated kinase and consequent ligand phosphorylation. To test this notion, Cos-1 cells expressing Elk-L or Htk-L were co-cultured with the neuronal cell line NG108-15[20] (NG108), that does not express endogenous Eph receptors which bind TM-ligands, or with a transfected NG108 clone which stably expresses high levels of the 130 kDa mouse Nuk protein (NG108-Nuk). In cocultures of ligand-expressing cells with NG108-Nuk cells, both the induction of Nuk tyrosine phosphorylation, reflecting activation of the Nuk catalytic domain, and also tyrosine phosphorylation of Elk-L or Htk-L, were observed which is consistent with stimulation of a ligand-associated tyrosine kinase in the ligand-expressing cells (Figure 2C). Parental NG108 cells lacking Nuk were without effect and conversely, no phosphorylation of either TM-ligands or Nuk was induced using untransfected Cos-1 cells (Figure 2C and data not shown).

The observation that Elk-L and Htk-L are inducibly phosphorylated on tyrosine in cultured cells upon exposure to clustered Nuk-Fc, or Nuk-expressing cells, suggests that this may be a physiological event. To test this possibility, protein lysates from mouse embryos at 10.5 days of development were immunoprecipitated with antibodies to either TM-ligands or Nuk, and the immune complexes were immunoblotted with antibodies to phosphotyrosine (Figure 3). Nuk immunoprecipitated from embryonic body or head tissue was phosphorylated on tyrosine (Figure 3 and data not shown). Furthermore, anti-ligand antibodies specifically

precipitated phosphotyrosine-containing polypeptides from these embryonic lysates that co-migrated with authentic TM-ligands. The intensity of the tyrosine phosphorylated band immunoprecipitated by the anti-ligand antibodies was markedly reduced by addition of excess ligand C-terminal peptide. These data demonstrate that not only Eph receptors such as Nuk, but also their TM-ligands, are phosphorylated on tyrosine in the developing mouse embryo.

Eph receptors and their ligands are expressed in reciprocal, mutually exclusive domains in the developing embryo[11]. Such expression patterns support data implicating Eph receptors in establishing boundaries between two distinct cell types, for example in the rhombomeres of the hindbrain and in development of the forebrain[9, 10]. To achieve this purpose, it would be advantageous if cell-cell contact initiated a bi-directional signal, thereby regulating the phenotype of both receptor- and ligand-expressing cells. The experiments described above demonstrated a biochemical mechanism through which such bi-directional signalling can be achieved (Figure 4). In the neuronal cell line NG108, activation of Nuk by TM-ligands leads not only to Nuk autophosphorylation, but also the phosphorylation of potential receptor targets (Figure 2C). The data also indicate that binding of Nuk to TM-ligands activates a tyrosine kinase in the ligand-expressing cell, leading to phosphorylation of the conserved C-terminal region of the ligand itself. The TM-ligands contain several tyrosine residues in a favourable sequence context for phosphorylation by Src-like kinases[21]. One scheme consistent with the results is that phosphorylation of the ligands by Src-like kinases induces the binding of SH2-containing proteins which then transmit signals within the ligand-expressing cell. However, the finding that TM-ligands are highly phosphorylated on tyrosine in mouse embryos suggests that ligand signalling is a significant event in the intact organism.

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Below full citations are set out for the references referred to in the footnotes in the specification.

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WE CLAIM:

1. A method of modulating the biological activity of a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand comprising forming a complex between a purified and isolated oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the Elk-related receptor tyrosine kinase, and the transmembrane ligand expressed on the cell, thereby modulating the biological activity of the transmembrane ligand.

2. A method of affecting a pathway regulated by a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand, comprising forming a complex between a purified and isolated oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the Elk-related receptor tyrosine kinase, and a transmembrane ligand expressed on the cell, thereby affecting or modulating a pathway in the cell which is regulated by the transmembrane ligand.

3. A method for evaluating a substance for its ability to modulate the biological activity of a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand comprising the steps of:

(a) contacting an oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the Elk-related receptor tyrosine kinase; a transmembrane ligand which binds to the Elk-related receptor tyrosine kinase to form a receptor-ligand complex, wherein the transmembrane ligand is a transmembrane ligand for an Elk-related receptor tyrosine kinase expressed on a cell; and, a test substance, under conditions which permit the formation of receptor-ligand complexes;

(b) assaying for receptor-ligand complexes, free Elk-related receptor tyrosine kinase, or non-complexed transmembrane ligand, or for activation of the transmembrane ligand; and

(c) comparing to a control to determine if the substance inhibits or enhances the binding of the Elk-related receptor tyrosine kinase and transmembrane ligand, and thereby modulates the biological activity of the transmembrane ligand.

4. A method for identifying a substance which affects or modulates a pathway regulated by a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand, comprising the steps of:

(a) contacting an oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the Elk-related receptor tyrosine kinase; a transmembrane ligand which binds to the Elk-related receptor tyrosine kinase to form receptor-ligand complexes which activate a pathway regulated by a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand; and a test substance under conditions which permit the formation of receptor-ligand complexes;

(b) assaying for receptor-ligand complexes, free Elk-related receptor tyrosine kinase, or non-

complexed transmembrane ligand, or for activation of the transmembrane ligand; and

(c) comparing to a control to determine if the substance inhibits or enhances the binding of the Elk-related receptor tyrosine kinase and transmembrane ligand, and thereby affects or modulates the pathway.

5. A method for evaluating a substance for its ability to inhibit or enhance the interaction of an oligomerized Elk-related receptor tyrosine kinase, or an isoform, or an extracellular domain of the Elk-related receptor tyrosine kinase, and a transmembrane ligand for an Elk-related receptor tyrosine kinase expressed on a cell comprising:

(a) providing a reporter gene operably linked to a DNA binding site for a transcriptional activator;

(b) providing a first hybrid protein comprising the transmembrane ligand in polypeptide linkage to a DNA binding domain of the transcriptional activator;

(c) providing a second hybrid protein comprising an oligomerized Elk-related receptor tyrosine kinase or an isoform or an extracellular domain of the Elk-related receptor tyrosine kinase in polypeptide linkage to an activation domain of the transcriptional activator; under conditions where the transmembrane ligand and oligomerized Elk-related receptor tyrosine kinase can bind and thereby reconstitute the transcriptional activator which induces transcription of the reporter gene;

(d) administering a test substance; and

(e) monitoring expression of the reporter gene, wherein a decrease in expression is an indication that the substance inhibits the interaction of the transmembrane ligand and oligomerized Elk-related receptor tyrosine kinase, and an increase in expression is an indication that the substance enhances the interaction of the transmembrane ligand and oligomerized Elk-related receptor tyrosine kinase.

6. A method for evaluating a substance for its ability to inhibit or enhance the interaction of an oligomerized Elk-related receptor tyrosine kinase, or an isoform, or an extracellular domain of the Elk-related receptor tyrosine kinase, and a transmembrane ligand for an Elk-related receptor tyrosine kinase expressed on a cell comprising:

(a) providing a reporter gene operably linked to a DNA binding site for a transcriptional activator;

(b) providing a first hybrid protein comprising an oligomerized Elk-related receptor tyrosine kinase or an isoform or an extracellular domain of the Elk-related receptor tyrosine kinase in polypeptide linkage to a DNA binding domain of the transcriptional activator;

(c) providing a second hybrid protein comprising a transmembrane ligand in polypeptide linkage to an activation domain of the transcriptional activator; under conditions where the transmembrane ligand and oligomerized Elk-related receptor tyrosine kinase can bind and thereby reconstitute the transcriptional activator which induces transcription of the reporter gene;

(d) administering a test substance; and

(e) monitoring expression of the reporter gene, wherein a decrease in expression is an indication that the substance inhibits the interaction of the transmembrane ligand and oligomerized Elk-related receptor tyrosine kinase, and an increase in expression is an indication that the substance enhances the interaction of

the transmembrane ligand and oligomerized Elk-related receptor tyrosine kinase.

7. A method as claimed in any one of claims 1 to 6, wherein the transmembrane ligand is Elk-L/LERK2/Efl-3/Cek5-L, hHtk-L/ELF-2/Lerk5, or hElk-L3/Efl-6.

8. A method as claimed in any one of claims 1 to 7, wherein the Elk-related receptor tyrosine kinase is Nuk , Hek5, Erk, Sek3, Cek5, Elk, Cek6a, xEK, Hek2, Sek4, Cek10, Htk, or Myk1.

9. A method as claimed in any one of claims 1 to 6 wherein the transmembrane ligand is Elk-L and the Elk-related receptor tyrosine kinase is Elk or Nuk.

10. A method as claimed in any one of claims 1 to 6 wherein the transmembrane ligand is Htk-L and the Elk-related receptor tyrosine kinase is Elk or Nuk.

11. An antibody preparation which specifically binds to a receptor-ligand complex comprising an oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain of the kinase, and a transmembrane ligand for an Elk-related receptor tyrosine kinase.

12. Use of a purified and isolated oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain thereof, in the preparation of a medicament for modulating the biological activity of a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand.

13. Use of a purified and isolated oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain thereof, in the preparation of a medicament for modulating neuronal development or regeneration in a subject.

14. Use of a purified and isolated oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain thereof, in the preparation of a medicament for modulating axonogenesis in a subject .

15. A use as claimed in any one of claims 12 to 14, wherein the transmembrane ligand is Elk-L/LERK2/Efl-3/Cek5-L, hHtk-L/ELF-2/Lerk5, or hElk-L3/Efl-6.

16. A use as claimed in any one of claims 12 to 15, wherein the Elk-related receptor tyrosine kinase is Nuk, Hek5, Erk, Sek3, Cek5, Elk, Cek6a, xEK, Hek2, Sek4, Cek10, Htk, or Myk1.

17. A use as claimed in any one of claims 12 to 14, wherein the transmembrane ligand is Elk-L and the Elk-related receptor tyrosine kinase is Elk or Nuk.

18. Use of a substance identified in accordance with a method as claimed in any one of claims 1 to 10 in the preparation of a medicament for modulating the biological activity of a transmembrane ligand for an Elk-related receptor tyrosine kinase in a cell expressing the transmembrane ligand.

19. A pharmaceutical composition which comprises a purified and isolated oligomerized Elk-related receptor tyrosine kinase, or an isoform or an extracellular domain thereof, in an amount effective to stimulate or inhibit neuronal development or regeneration and a pharmaceutically acceptable carrier, diluent or excipient.

FIGURE 1A

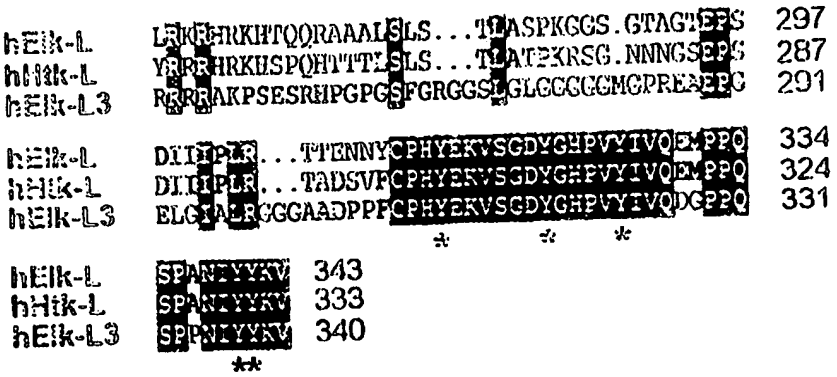

FIGURE 1B

FIGURE 1C

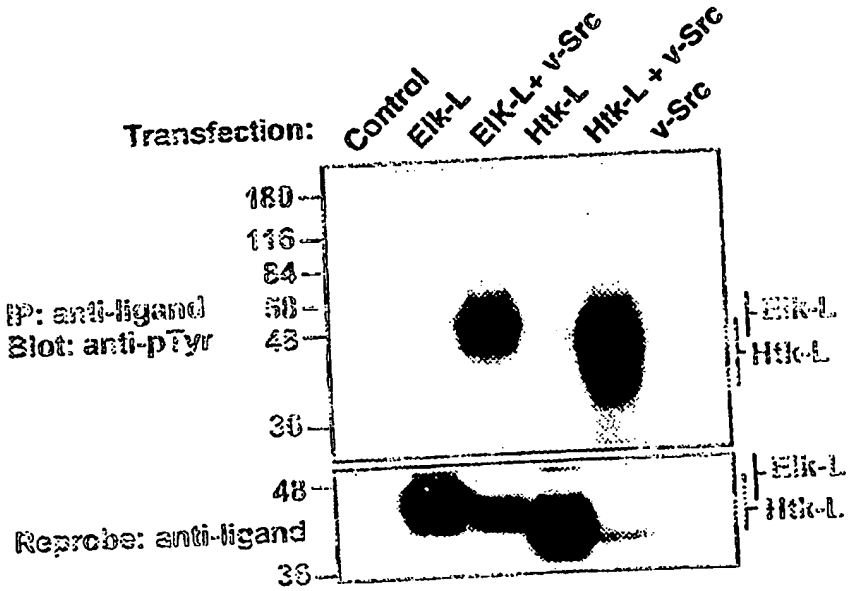
Transfection:
Control
Elk-L
Elk-L + v-Src
Htk-L
Htk-L + v-Src
v-Src
180
116
84
58
48
36
IP: anti-ligand
Blot: anti-pTyr
Elk-L
Htk-L
48
36
Reprobe: anti-ligand
Elk-L
Htk-L

FIGURE 1D

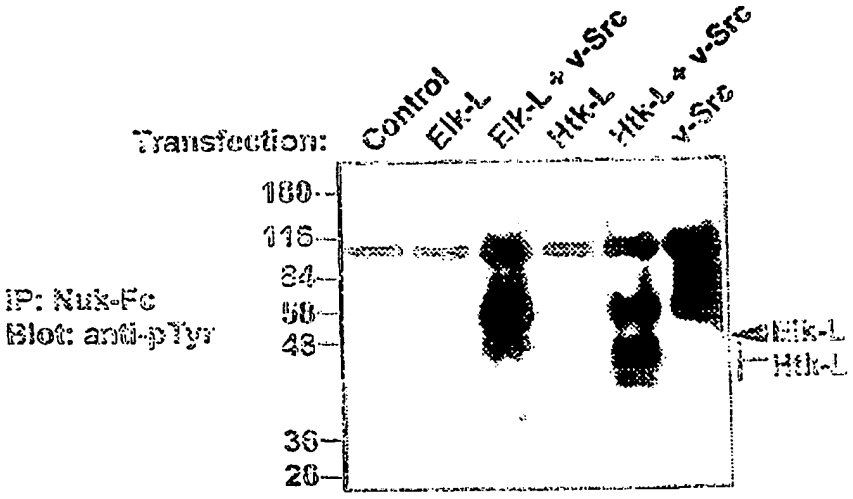
Transfection:
Control
Elk-L
Elk-L + v-Src
Htk-L
Htk-L + v-Src
v-Src
IP: Nuk-Fc
Blot: anti-pTyr
180
116
84
58
48
36
26
Elk-L
Htk-L

FIGURE 2A

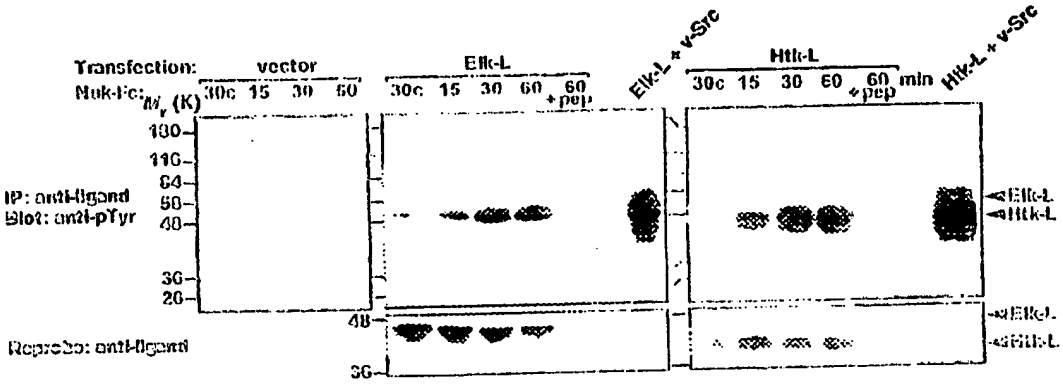
Transfection:
vector
Elk-L
Elk-L + v-Src
Htk-L
Htk-L + v-Src
Nuk-Fc: M_r (K)
30c 15 30 60
30c 15 30 60 60 +pep
30c 15 30 60 60 +pep min
180
116
84
58
48
36
26
IP: anti-ligand
Blot: anti-pTyr
Reprobe: anti-ligand
Elk-L
Htk-L

FIGURE 2B

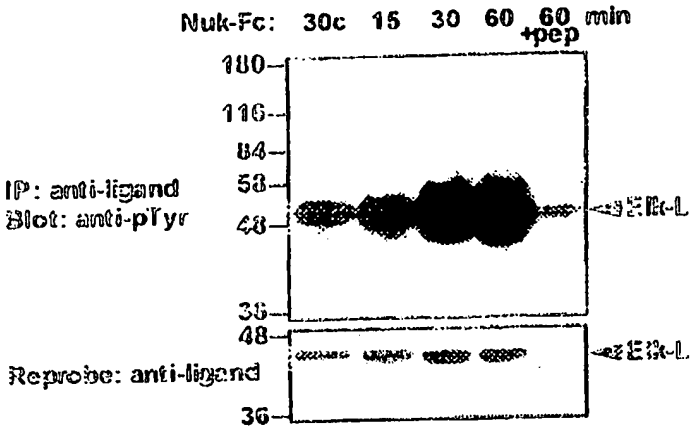
Nuk-Fc: 30c 15 30 60 60 min
+pep
180
116
84
58
48
36
IP: anti-ligand
Blot: anti-pTyr
EPh-L
48
36
Reprobe: anti-ligand
EPh-L

FIGURE 2C

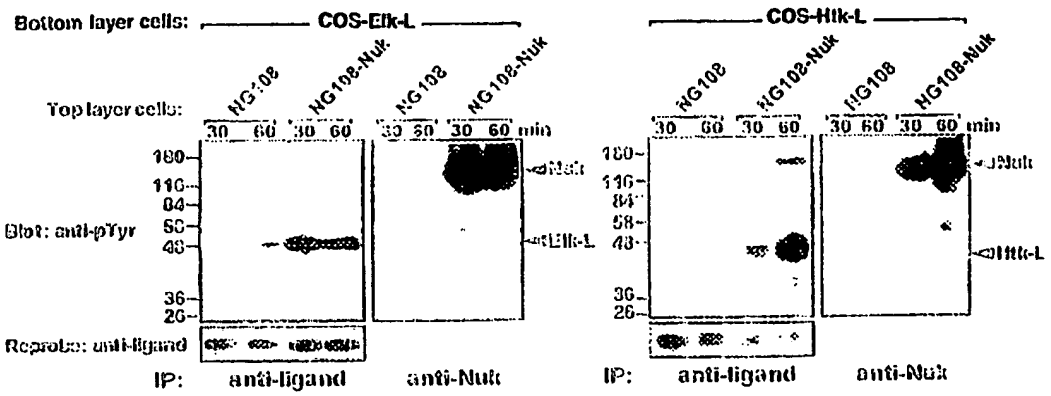
Bottom layer cells:
COS-Elk-L
COS-Hlk-L
Top layer cells:
NG108
NG108-Nuk
NG108
NG108-Nuk
NG108
NG108-Nuk
NG108
NG108-Nuk
30 60 30 60 30 60 30 60 min
30 60 30 60 30 60 30 60 min
180
116
84
58
48
36
26
Blot: anti-pTyr
Reprobe: anti-ligand
Nuk
Elk-L
Hlk-L
IP: anti-ligand anti-Nuk
IP: anti-ligand anti-Nuk

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FIGURE 3

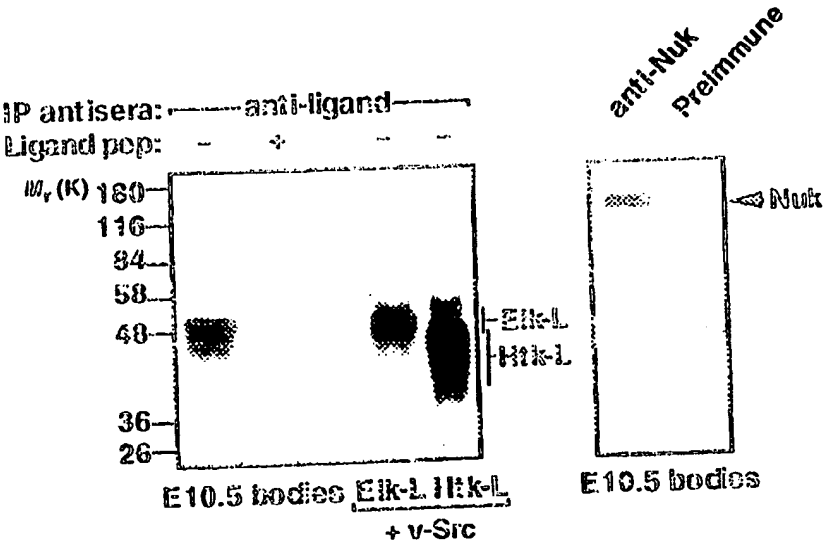

IP antisera:
anti-ligand
Ligand pep:
anti-Nuk
Preimmune
M_r (K)
180
116
84
58
48
36
26
Elk-L
Htk-L
Nuk
E10.5 bodies Elk-L Htk-L
+ v-Src
E10.5 bodies

FIGURE 4

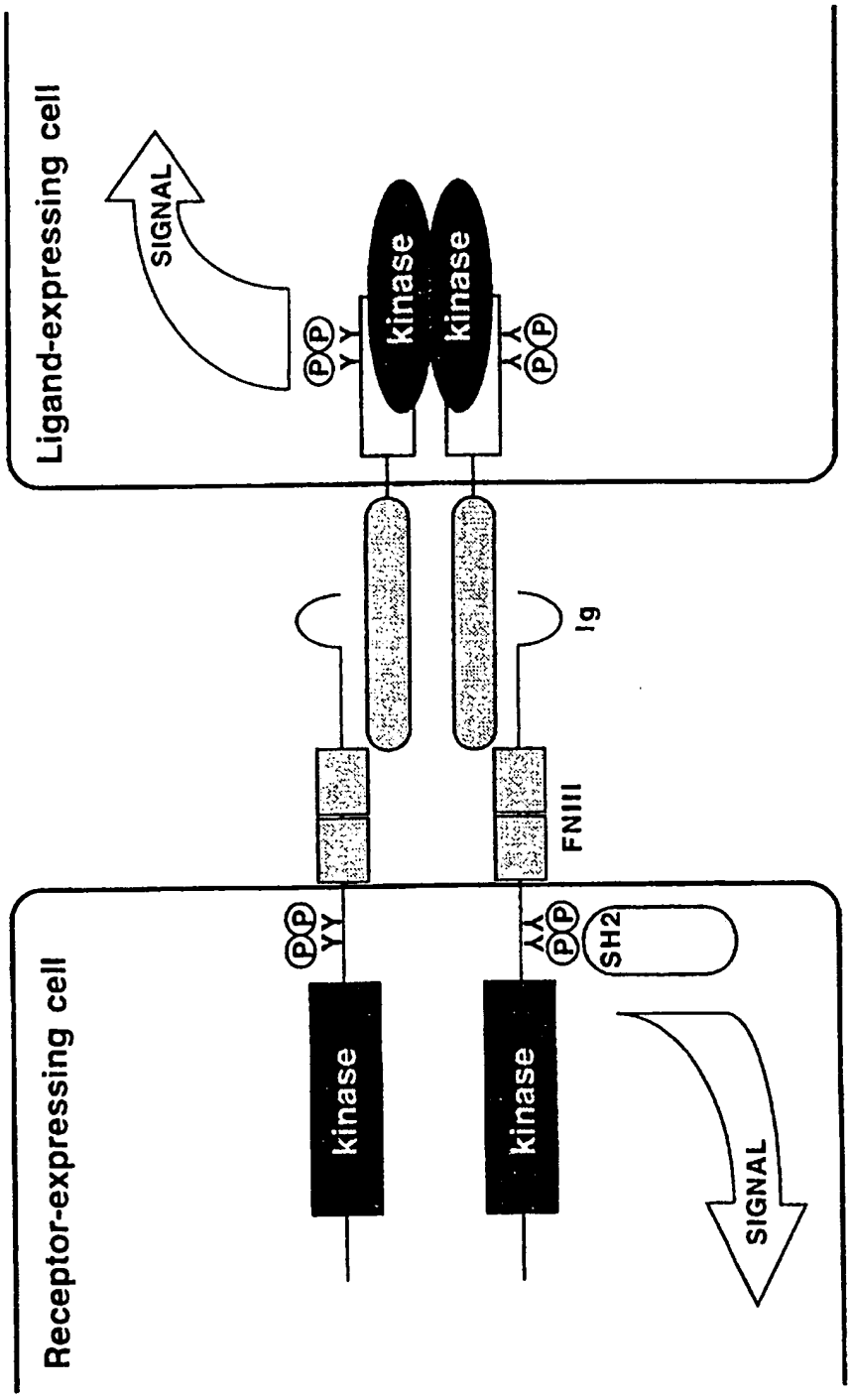

Ligand-expressing cell
SIGNAL
kinase
kinase
P P
Y Y
Y Y
P P
Ig
FNIII
P P
Y Y
Y Y
P P
SH2
kinase
kinase
Receptor-expressing cell
SIGNAL

FIGURE 5A

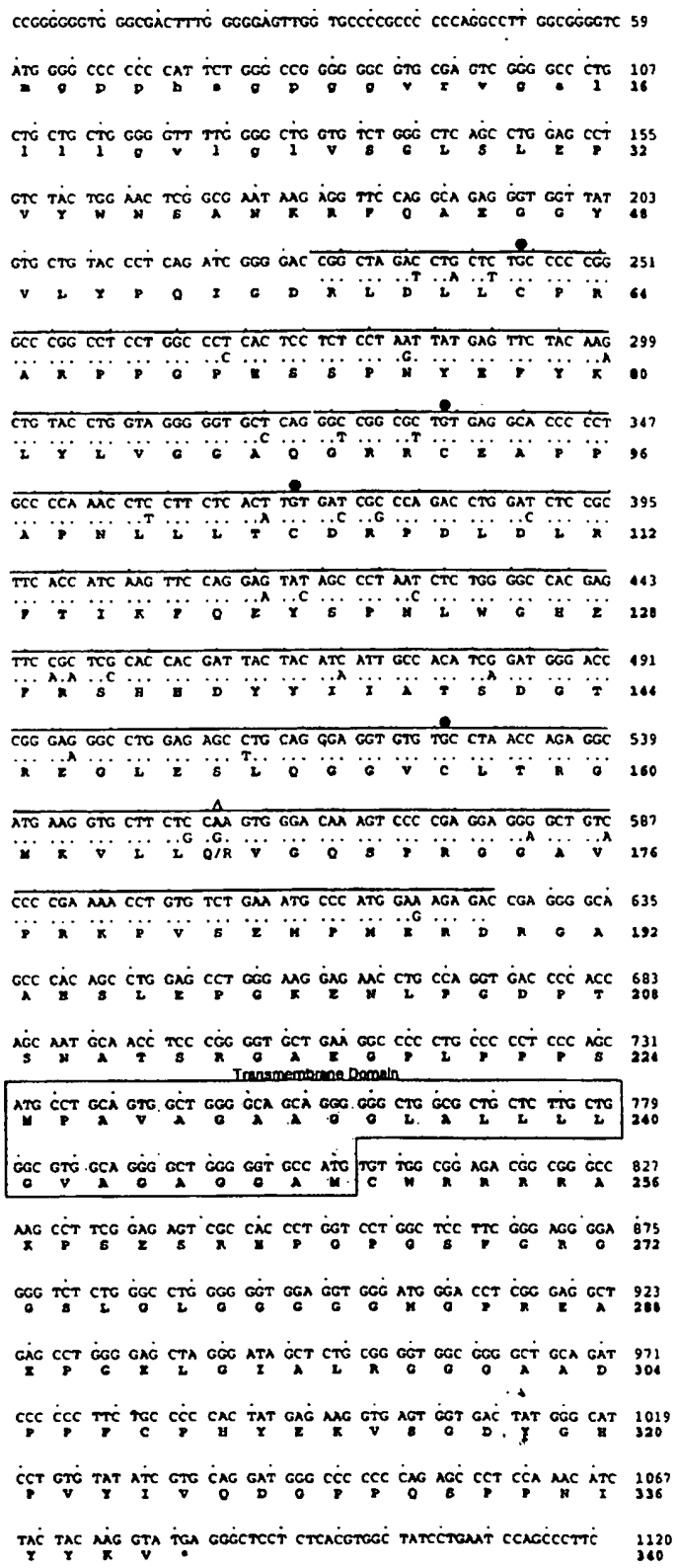


FIGURE 5B

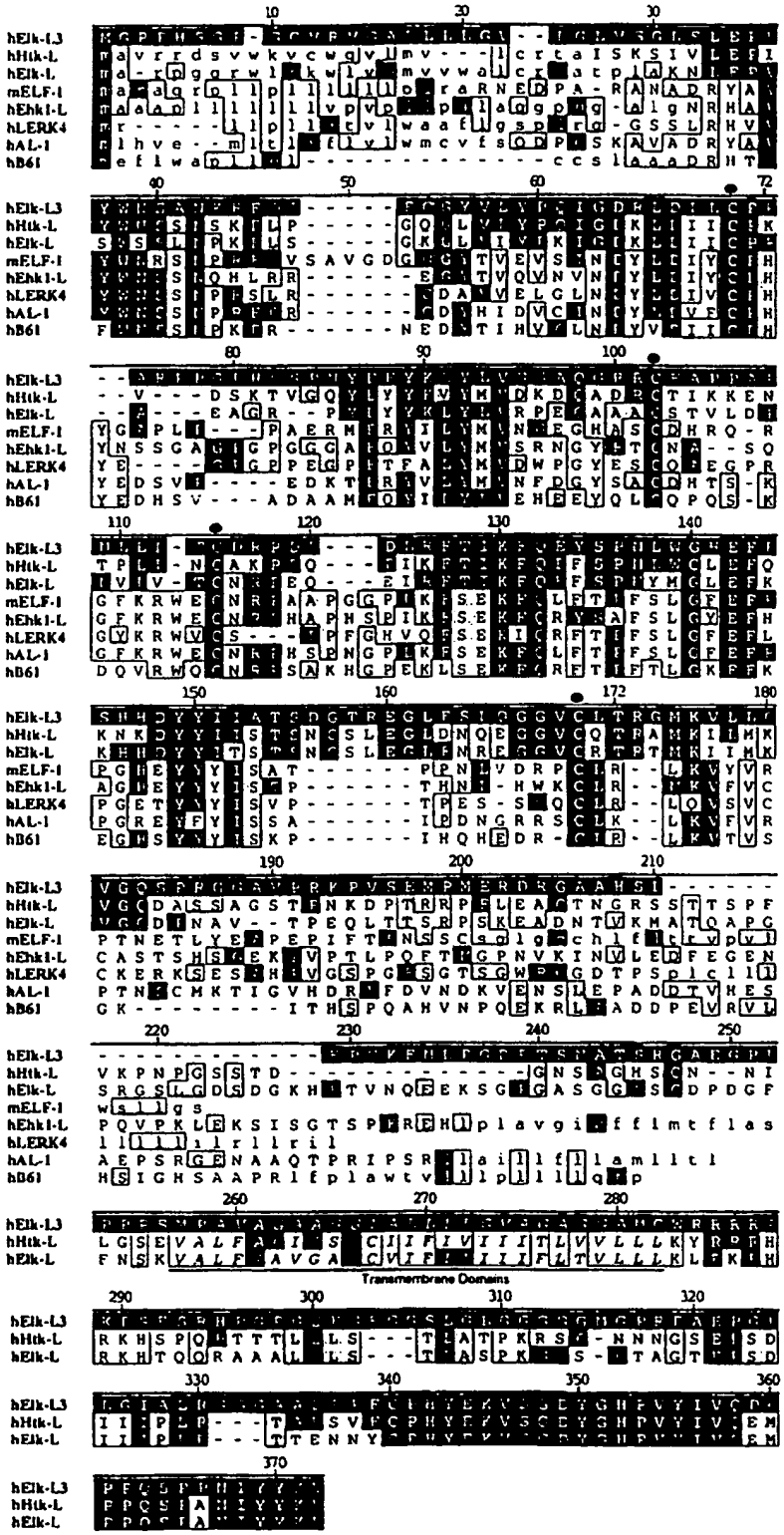
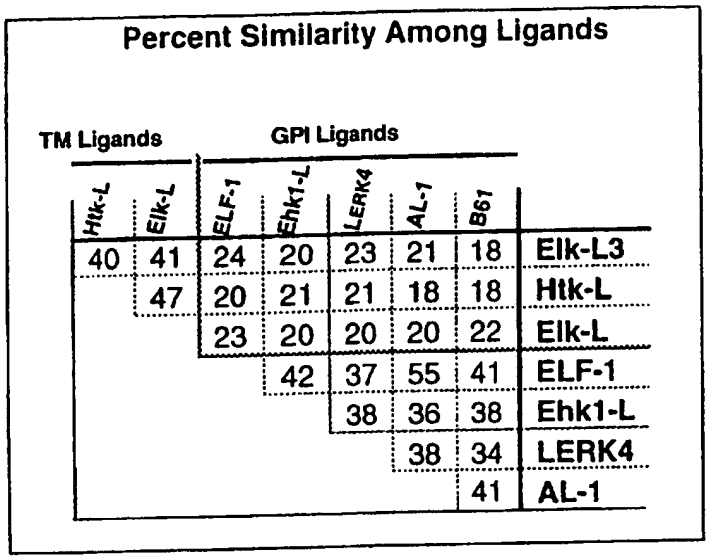

hElk-L3
hHtk-L
hElk-L
mELF-1
hEhk1-L
hLERK4
hAL-1
hB61
Transmembrane Domains

FIGURE 5C


Percent Similarity Among Ligands
TM Ligands
GPI Ligands
Htk-L
Elk-L
ELF-1
Ehk1-L
LERK4
AL-1
B61
40 41 24 20 23 21 18 Elk-L3
47 20 21 21 18 18 Htk-L
23 20 20 20 22 Elk-L
42 37 55 41 ELF-1
38 36 38 Ehk1-L
38 34 LERK4
41 AL-1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 97/00473

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/10 C12N15/62 C12N9/12 C07K14/47
C07K14/705 C07K16/18 C12Q1/68 A61K38/43 G01N33/50
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K C12Q A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 27060 A (REGENERON PHARMA) 12 October 1995 see page 32, line 13 - page 33, line 15; example 1 ---	1,2
X	WO 95 30326 A (MOUNT SINAI HOSPITAL CORP ;PAWSON ANTHONY (CA); HENKEMEYER MARK (C) 9 November 1995 see the whole document --- -/--	1-4

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 December 1997	19/12/1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 97/00473

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GALE, NICHOLAS W. ET AL: "Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis" NEURON (1996), 17(1), 9-19 CODEN: NERNET;ISSN: 0896-6273, 1 July 1996, XP002048874 cited in the application see the whole document	1-19
A	WO 93 00425 A (INST MEDICAL W & E HALL) 7 January 1993 see claims 11-28	1-19
A	WO 94 11384 A (IMMUNEX CORP) 26 May 1994 see page 4, line 9 - line 20 see examples 1,7	1-19
A	WO 95 06065 A (IMMUNEX CORP) 2 March 1995 see example 5	1-19
A	WO 95 28484 A (AMGEN INC) 26 October 1995 see page 11, line 33 - page 14, line 26	1-19
A	WO 96 01839 A (IMMUNEX CORP) 25 January 1996 see the whole document	1-19
A	S. DAVIS ET AL.: "Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity" SCIENCE, vol. 266, 4 November 1994, AAAS,WASHINGTON,DC,US, pages 816-819, XP002038222 cited in the application see the whole document	1-19
P,X	WO 97 14966 A (MOUNT SINAI HOSPITAL CORP) 24 April 1997 see the whole document	1-4, 12-19
P,A	WO 96 26958 A (HARVARD COLLEGE) 6 September 1996 see the whole document	1-19
T	S.J. HOLLAND ET AL.: "Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands" NATURE, vol. 383, 24 October 1997, MACMILLAN JOURNALS LTD., LONDON,UK, pages 722-725, XP002048843 see the whole document	1-19

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Information on patent family members

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PCT/CA 97/00473

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		ZA 9502762 A	20-02-96
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		CA 2169851 A	02-03-95
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		CA 2189028 A	26-10-95
		EP 0756627 A	05-02-97
WO 9601839 A	25-01-96	AU 3094095 A	09-02-96
		CA 2194299 A	25-01-96

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 97/00473

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9601839 A		EP 0770088 A	02-05-97
		FI 965297 A	07-03-97
		NO 965627 A	07-03-97
WO 9714966 A	24-04-97	NONE	
WO 9626958 A	06-09-96	NONE	